Development of an in vitro method for detection of Clostridium botulinum types A and E using real-time PCR

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Summary: Specific primers for *C. botulinum* types A and E neurotoxin genes were evaluated both from the literature and of own construction. Furthermore, a real-time system with specific hybridisation probes was designed for *C. botulinum* type E neurotoxin gene, and is under construction for type A. Sequencing of part of the neurotoxin gene for type E showed some differences with sequences reported in GenBank. The PCR methods were optimised regarding amplification efficiency, linear range and specificity. The detection limit for type E using real-time PCR is at least 0,1 ng/ml, corresponding to 0,4 pg of total DNA in the tube, and at least 0,5 ng/ml (2,5 pg of total DNA) for type A using conventional PCR. Quantitative reverse transcription PCR was used to study the relative expression of the neurotoxin gene in different growth phases.

Keywords: Botulism, neurotoxin, molecular methods, relative quantification, gene expression

Introduction: *C. botulinum* types A, B and E are the most common causes of foodborne botulism in humans. They are a diverse group of organisms that have different growth characteristics and nutritional requirements. The diagnosis of botulism is usually established by detecting the neurotoxin in serum, stomach content, faeces and suspected foods using the mouse bioassay. This method is, however, expensive, relatively slow and raises moral issues about animal rights. The use of PCR has been found to be an accurate and sensitive way to determine the presence of different foodborne pathogens. The ability to quickly and easily detect the bacteria and its neurotoxin would increase the possibility of tracing the contamination back to its source. A fast, specific and sensitive *in vitro* method would also be of great help in securing microbiological food safety. The goals of this project are to develop methods that could be used for the detection and enumeration of *C. botulinum* in food-, faecal- and patient samples, as well as to study how environmental factors affect the neurotoxin production.

Materials and methods: DNA was purified from overnight cultures using the EasyDNA kit (Invitrogen). For gram-positive strains 8 mg/ml of lysosyme and 40 U/ml of mutanolysine were added and the sample was incubated at 37AC for 30 min before the purification. Several different primer pairs targeting the *bont* genes of *C. botulinum* types A and E were evaluated regarding specificity, sensitivity and reproducibility. The specificity of the PCR assay was evaluated using DNA from 8 strains of *C. botulinum* type A, 16 strains of *C. botulinum* type E, 16 strains of *C. botulinum* types B and F, 16 strains of other *Clostridium* spp., and 26 non-*Clostridium* strains. Conventional PCR was carried out on a Gene Amp 9700 thermal cycler in a total volume of 25 ml. For type A the primers and protocol were modified from Takshi et al. (1996). For type E the PCR mixture contained 1X PCR Buffer, 0.2 mM of each dNTP 0.5 mM of each primer, 1.25 U Taq DNA Polymerase, and 5 ml of template solution. The amplification commenced with a denaturation step at 94AC for 7 min, followed by 40 cycles consisting of heat denaturation at 94AC for 40 s, primer annealing at 58AC for 40 s, and extension at 72AC for 40 s. Finally extension was performed at 72AC for 7 min to complete the synthesis of all strands. The products were visualized by agarose gel electrophoresis. DNA-sequencing of the type E amplicon was performed at the BM-unit, Lund University, according to the chain-termination method (Sanger

et al. 1977). Several different hybridisation probes were tested for the *bont* E gene and a real-time PCR assay, using the LightCycler™ instrument, was developed. The hybridisation probes consist of two parts; a donor probe labelled with fluorescein at the 3'-end and an acceptor probe labelled with LC Red640 (LC) at the 5'-end and 3'-hydroxy blocked with a phosphate. The PCR mixture contained 1X PCR Buffer, 4 mM $MgCl_2$, 0.2 mM of each dNTP, 0.3 mM of each primer, 0.3 mM of each probe, 1.25 U Tth DNA Polymerase, and 4 ml of template solution. The total volume added to each capillary was 20ml. The amplification protocol used started with an initial denaturation at 95AC for 60 s, followed by 45 cycles of 95AC for 0s, annealing and fluorescence acquisition at 56AC for 5 s, and elongation at 72AC for 25 s. Primers and probes have also been developed for the *rrn* gene, a housekeeping gene, to allow relative quantification of gene expression. A modified method for total RNA extraction from *Bacillus* spp. with acidic phenol was used (Putzer et al., 1992). First-strand cDNA was synthesized in two separate RT assays using the reverse primers for the *bont* E gene and the *rrn* gene. cDNA synthesis was performed in a Gene Amp 9700 thermal cycler. The total volume of the reaction mixture was 20 µl and contained 0.1 µg total RNA, 0.5 mM of each primer, 5 mM of each nucleotide dATP, dTTP, dCTP and dGTP, 20 U RNasin® ribonuclease inhibitor, 5 mM DTT, 1¥ first-strand buffer and 200 U Superscript™ II RNase H^- reverse transcriptase. Before RT enzymes were added, the reaction mixture was heated to 65°C for 5 min and thereafter chilled on ice. After brief centrifugation and the addition of RT enzymes, the reaction mixture was incubated at 42°C for 50 min and the reaction was terminated by incubation at 70°C for 15 min. The real-time PCR protocol described above was used, but using 0.5 mM of the primers for the *rrn* gene.

Results and discussion: All 8 strains of *C. botulinum* type A could be detected using the conventional PCR, while none of the other strains gave any bands. The detection limit was at least 0.5 ng/ml, corresponding to 2.5 pg of total DNA in the tube. During the comparison of primers and probes some anomalies were detected among the type E strains. Therefore the amplicons were sequenced. A difference from the sequences reported in GenBank was found in two of the strains. All of the tested *C. botulinum* type E strains could be detected using the method and none of the other strains gave any detection. The detection limit for the real-time assay was at least 0.1 ng/ml, which corresponds to 0.4 pg of total DNA in the capillary. When following the relative expression of two different strains of *C. botulinum* type E it was found that the expression of the neurotoxin was dependent on when in the growth cycle the sample was taken. Preliminary results show that the neurotoxin gene expression increases during the exponential phase, reaching its maximum as the bacteria enter in the stationary growth phase. This agrees with previous findings for other *C. botulinum* type E strains [McGrath, 2000], as well as with *C. botulinum* type B (Lövenklev et al. manuscript).

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